

**ATTACHMENT E**

IN THE MATTER OF  
US Patent Application No. 09/380,327  
by ROBERTSON et al.

**Declaration under U.S.C. § Rule 132**

**SECOND STATUTORY DECLARATION**

I, David Alexander Clark of 444 Smith Ave., Burlington in the Province of Ontario, Canada, do solemnly and sincerely declare as follows:

1. \_\_\_\_\_ I am the same David Alexander Clark who made a statutory declaration dated 16 December 2002 in respect of this matter.

2. \_\_\_\_\_ I have been provided with copies of the following documents:

Abstract by Tremellen K.P. and Robertson S.A. entitled "Potential role for transforming growth factor beta in modulating the maternal immune response during early murine pregnancy" published by The Australian Society for Medical Research on 24-27 November 1996; and

Abstract by Tremellen K.P. and Robertson S.A. entitled "Isolation of seminal vesicle proteins responsible for the initiation of the post-mating inflammatory response" published by The Australian Society for Medical Research on 31 May 1996.

Copies of these two abstracts are annexed hereto as Exhibit DC-1.

3. \_\_\_\_\_ I have been informed that both of these abstracts were published before the priority date of the present application, 6 March 1997, and have only recently come to the attention of the licensee of the present application, who is responsible for instructions in relation to this case.

4. \_\_\_\_\_ I have also been provided with a copy of the paper by Robertson et al., "Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice" (J Reproduction and Fertility (1996) 107, 265-277), and the abstract of a paper by Robertson and Seamark entitled "Granulocyte macrophage colony stimulating factor (GM-CSF) in the murine reproductive tract: stimulation by seminal factors" (Reprod Fertil Dev, (1990) 2(4): 359-68). The paper by Robertson et al is referred to in the specification at page 4 lines 18 to 22, and is reference 10 listed at page 30 of the specification. The paper by Robertson and Seamark is reference 8 cited at page 4 line 21 of the specification, and is reference 8 in the list at page 30. Copies of these documents are annexed hereto as Exhibits DC-2 and DC-3.

5. \_\_\_\_\_ I am aware that it was known before the priority date that human seminal fluid contains high levels of TGF $\beta$ . This is disclosed in the paper by Nocera and Chu (1995) (Am. J. Reprod. Immunol. 33, 282-291), which is also referred to in the specification and is reference 22 in the list at page 30.

6. \_\_\_\_\_ I have read and understood the two abstracts, and I do not consider that they convey any significant further information over and above the disclosures of the reference which are discussed at page 4 lines 14 to 29 of the specification.

7. \_\_\_\_\_ Nor do I consider that they provide any significant information over and above any of the references previously cited by the Examiner, and which have been discussed in my earlier declarations in this matter.

8. \_\_\_\_\_ I do not consider that either of these abstracts would lead a person of ordinary skill in the art as at the priority date, 6 March 1997, to consider that treatment with TGF $\beta$  would be useful in the treatment of an infertility condition as defined in the specification, including recurrent miscarriage or spontaneous abortion. The abstracts show that TGF $\beta$  elicited a non-specific inflammatory response. At the priority date inflammation was regarded as having an *anti*-fertility effect. For example, intrauterine devices were reported to exert a contraceptive effect by eliciting intrauterine inflammation. In the condition called endometriosis, release of increased levels of proinflammatory cytokines such as TNF- $\alpha$  and interferon- $\gamma$  was regarded as *preventing* spermatozoa from fertilizing oocytes, preventing the normal division of the fertilized ovum, and preventing normal outgrowth of the embryonic trophoblast cells which is required for attachment of

the fertilized ovum to and invasion of the endometrium. Similarly, stimulation of release of proinflammatory cytokines by bacterial lipopolysaccharide (endotoxin) was a well-known cause of abortions.

9. \_\_\_\_\_ I consider that the two abstracts and the earlier publications of Robertson et al and Robertson and Seamark would have led the person skilled in the art to consider that TGF $\beta$  would have an anti-fertility effect, and would cause rather than prevent abortion.

10. \_\_\_\_\_ I note that the May abstract refers to a primary role for seminal TGF $\beta$ 1 in initiating the post-mating inflammatory response in mice, while the November abstract refers to "investigation of the potential role of this response in generating a tolerogenic maternal immune response conducive to growth and survival and the semi-allogenic (sic) embryo during pregnancy." In the 1996 paper by Robertson et al (reference 10 in the specification) it was not clear how a proinflammatory stimulus, which was subsequently identified in the abstract as TGF $\beta$ , could promote development of immunological tolerance. At lines 10 to 11 of the introduction to that paper, the authors suggest that the hormone progesterone, not the proinflammatory stimulus, was responsible for causing a switch to a tolerogenic state. At the priority date, even though it was known that TGF $\beta$  had immunosuppressive properties, as well as many other biological properties, because the effects of plasma TGF $\beta$  had been shown in the abstracts to be proinflammatory, they would not be suspected of inducing a tolerance response.

11. \_\_\_\_\_ Furthermore, the meaning of the expression "a tolerogenic maternal immune response conducive to the growth and survival of the semi-allogeneic embryo during pregnancy" is far from clear. The state of tolerance is a grossly observable phenomenon which may arise via one or more distinct underlying mechanisms. To add to the conceptual confusion prevailing in 1997, some investigators had been proposing that an active immune response, even immunostimulation, was required for successful pregnancy, and in the light of these proposals immunosuppression and "tolerance", meaning absence of an immune response, would not clearly lead to enhanced success of pregnancy.

12. \_\_\_\_\_ I therefore consider that a considerable amount of further work would have been required before any possible therapeutic application on the basis of the observations described in the

abstracts could have been suggested. Consequently I consider that the two abstracts teach away from the claimed invention, and at most constitute an invitation for further experimentation.

I declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by prison or fine or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any application or patent thereon.

DECLARED at                      this                      day of                      2003

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David A. Clark

Before me:

A person empowered to witness Statutory  
Declarations under the laws of the Province of  
Ontario, Canada.

**EXHIBIT DC-1**



*The Australian Society  
for  
Medical Research*

*35th National  
Scientific Conference*

*ANA Hotel, Gold Coast, Qld  
24th-27th November, 1996*

**PROGRAMME  
AND  
ABSTRACTS**

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### Oral 3-3

## POTENTIAL ROLE FOR TRANSFORMING GROWTH FACTOR BETA IN MODULATING THE MATERNAL IMMUNE RESPONSE DURING EARLY MURINE PREGNANCY.

Kelton P. Tremellen, Sarah A. Robertson

Dept. Obstetrics and Gynaecology, University of Adelaide, South Australia 5005

Mating in the mouse evokes a local inflammatory reaction with extensive infiltration of the uterus with leucocytes, predominantly macrophages, granulocytes and dendritic cells. Previous work has shown that this response is initiated by Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) originating from the oestrus uterine epithelium. The trigger for the release of epithelial GM-CSF has been localised to the seminal vesicle-derived component of the ejaculate. In order to identify the active moiety, seminal vesicle secretions were fractionated according to molecular weight using size exclusion chromatography and then cultured with uterine epithelial cells. This revealed that the majority of the GM-CSF stimulating activity eluted in a heterogeneous peak of 150-400 kDa molecular weight, the activity of which was potentiated by acid activation. This activity was identified as Transforming Growth Factor Beta (TGFβ) since neutralising anti-TGFβ polyvalent antibody completely abrogated the GM-CSF stimulating capacity of the active fractions, and high levels of TGFβ (both bioactivity and immunoreactivity) were present in this 150-400 kDa molecular weight range. When TGFβ levels were measured in the secretions and tissues of the male accessory glands which produce seminal plasma, the seminal vesicle gland was found to be the major source of TGFβ (comprising 94% of total ejaculate TGFβ) with an average concentration of 70 ng/ml. The majority (70%) of this TGFβ was in the biologically inactive latent form, but the existence of mechanisms in the uterus for activation of latent TGFβ were suggested by the finding that TGFβ levels in the uterine luminal fluids increase 3-fold after mating, with the majority of TGFβ present in the biologically active form. Furthermore, injection of recombinant TGFβ into uteri of oestrus mice was found to produce both an increase in uterine fluid GM-CSF and a leucocyte influx comparable to that seen following natural mating. Together, these data show that TGFβ in seminal plasma initiates the post-mating inflammatory response in the murine uterus. Ongoing experiments are investigating the potential role of this response in generating a tolerogenic maternal immune response conducive to growth and survival of the semi-allogenic embryo during pregnancy.

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**SCIENTIFIC MEETING**

31st MAY, 1996

The Orphanage Teacher's Centre  
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**10\* Isolation of seminal vesicle proteins responsible for the initiation of the post-mating inflammatory response.**

Kelton Tremellen, Sarah Robertson, Dept. of Obstetrics and Gynaecology, University of Adelaide.

Mating in the mouse evokes a local inflammatory response with extensive infiltration of the endometrial stroma and uterine lumen with activated macrophages and granulocytes. This response has been suggested to be important for the generation of an immunological environment conducive to the survival of the semi-allogenic embryo. Previous studies have shown that granulocyte macrophage colony stimulating factor (GM-CSF) produced by the oestrus uterine epithelium in response to an unknown factor in seminal plasma triggers this inflammatory response. Experiments looking at the stimulatory capacity of secretions from the various male accessory sex glands and matings with male mice from which glands were surgically removed had shown the stimulatory factor(s) to be derived from the seminal vesicle. The aim of recent studies has been to further characterise this activity in seminal vesicle fluid.

Seminal vesicle fluid was separated on the basis of molecular weight using a Sephacryl S 400 FPLC column and each fraction was assayed for its GM-CSF stimulating capacity on uterine epithelial cells *in vitro*. Two distinct stimulating factors were identified; a high molecular weight protein of 650 kDa which was sensitive to trypsin, heat and acetone extraction, and a lower molecular weight fraction that eluted over the range of 150-400 kDa. This lower molecular weight fraction was found to have transforming growth factor beta (TGF $\beta$ ) bioactivity in a specific bioassay (MvLu cells) and was confirmed as TGF $\beta_1$  in an ELISA, and by the finding that rabbit anti-TGF $\beta_1$  polyvalent antibody neutralised the GM-CSF stimulating activity of seminal vesicle fluid.

Furthermore recombinant TGF $\beta_1$  has been shown to increase uterine epithelial cell GM-CSF production in both *in vitro* culture and after instillation into oestrus uteri *in vivo*. Injection of rTGF $\beta_1$  into the lumen of oestrus uteri was found to elicit an inflammatory reaction resembling that seen following natural mating. Together these findings indicate a primary role for seminal TGF $\beta_1$  in initiating the post-mating inflammatory response in mice.

**20\* MAST CELLS AND SUPPRESSION OF IMMUNE RESPONSES BY UVB IRRADIATION**

MA Grimbaldeston, GJ Swift, JJ Finlay-Jones &amp; PH Hart, Department of Microbiology &amp; Infectious Diseases, School of Medicine, Flinders University of South Australia.

UVB irradiation (280-320 nm) suppresses contact hypersensitivity responses in both mouse and man. In mice, three phenotypes with significantly different susceptibilities to UVB suppression have been identified. BALB/c mice require nearly 6 times more UV than C57 BL/6 mice for 50% suppression of contact hypersensitivity responses; these strains have been defined to have low and high susceptibilities to UV, respectively. DBA/2 mice have an intermediate susceptibility. Mast-cell-depleted mice (Wf/Wf) are unresponsive to UVB, but

**EXHIBIT DC-2**

## Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice

S. A. Robertson, V. J. Mau, K. P. Trémellen and R. F. Seamark

*Department of Obstetrics and Gynaecology, University of Adelaide, South Australia, 5005, Australia*

Mating evokes a characteristic pattern of molecular and cellular events in the rodent reproductive tract, including an infiltration of the endometrial stroma and uterine lumen with activated macrophages and granulocytes, which closely resembles a classic inflammatory response. Previous studies in mice indicate that these cellular changes are associated with, and are largely a consequence of, an upregulated synthesis and release of granulocyte-macrophage colony-stimulating factor (GM-CSF) from the uterine epithelium in response to seminal fluid. The aim of this study was to investigate further the origin and nature of the factors present in seminal fluid that trigger the GM-CSF response. It was found that the characteristic increase in uterine expression of mRNA encoding GM-CSF and release of GM-CSF bioactivity from uterine epithelial cells into the luminal cavity seen after mating with intact or vasectomized males was no longer evident in matings with male mice from whom the seminal vesicles had been surgically removed. The extent of inflammatory leucocyte infiltration into the endometrium was also reduced; the most notable effect was a complete absence of the exocytosis of neutrophils into the luminal cavity normally seen after matings with intact or vasectomized males. Bioassay of the GM-CSF output of oestrous endometrial cells after culture with crude or Sephacryl S-400 chromatographed fractions of seminal vesicle fluid showed that the GM-CSF stimulating activity was predominantly associated with protein moieties in seminal vesicle fluid of approximately 650 000  $M_r$  and 100 000–400 000  $M_r$ . These data confirm the presence in seminal vesicle fluid of specific factors that initiate an inflammatory response in the uterus after mating through upregulating GM-CSF synthesis in the uterine epithelium. The significance of the cytokine release and cellular changes induced by seminal plasma for implantation of the conceptus and pregnancy outcome remain to be determined.

### Introduction

Ejaculation during copulation initiates a cascade of cellular and molecular events required for pregnancy. Accumulating evidence implicates a 'priming' role for specific uterotrophic factors in seminal fluid. In rodents, seminal fluid infused into the uterus at oestrus incites a marked local inflammatory response, characterized by extensive infiltration of activated neutrophils, eosinophils and macrophages into the endometrial stroma and lumen (De *et al.*, 1991; Kachache *et al.*, 1991; McMaster *et al.*, 1992). This inflammatory response subsides by day 3 of pregnancy in response to a progesterone-induced shift in the local cytokine milieu (Kachache *et al.*, 1991), which appears to be accompanied by a transition in local macrophages to an 'immunosuppressive' phenotype, conducive to the implantation and development of the semi-allogeneic embryo (Hunt *et al.*, 1984).

The inflammatory reaction after mating is preceded by a transient, 20-fold increase in the content of the lymphohaemopoietic cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) in luminal fluid. This increase occurs within

12 h after mating and is induced by seminal factors acting on the oestrogen-primed epithelium (Robertson and Seamark, 1990; Robertson *et al.*, 1992). Accumulation of GM-CSF bioactivity at the epithelial surface is implicated as a key intermediary in the local recruitment and activation of inflammatory leucocytes, as the introduction of equivalent amounts of recombinant GM-CSF into the uterine lumen of an ovariectomized mouse is sufficient to cause an infiltration of macrophages, neutrophils and eosinophils, indistinguishable from that seen on day 1 of pregnancy (Robertson *et al.*, 1994) and the inflammatory response after mating is diminished in GM-CSF deficient transgenic mice (S. Robertson, unpublished). The experiments described in this study were aimed at exploring the origin and nature of the factor(s) in seminal fluid that initiates the release of GM-CSF from oestrogen-primed epithelial cells.

### Materials and Methods

#### *Animals and surgical procedures*

Adult (8–12 week) (Balb/c  $\times$  C57B1)  $F_1$  female mice and (CBA  $\times$  C57B1)  $F_1$  male mice obtained from the University of

Received 5 January 1996.

Adelaide Central Animal House, were maintained in minimal security barrier, specific pathogen free facilities, on a 12 h light:12 h dark cycle with food and water available *ad libitum*. Adult, naturally cyclic female mice were examined daily and vaginal smears were taken to identify mice in oestrus, or were placed two females per cage with individual males for natural mating. The day a vaginal plug was observed was nominated as day 1 of pregnancy or pseudopregnancy after mating with intact or vasectomized males. In matings using seminal vesicle-deficient males, vaginal smears were taken each morning and checked for the presence of spermatozoa, since a copulatory plug was not formed. Uterine tissues and fluids from females mated with seminal vesicle-deficient males were included in analyses only if the sperm content of uterine luminal fluids was  $> 10^6 \text{ ml}^{-1}$  (approximately 50% of mated mice). In one experiment, male mice received a single s.c. injection of testosterone (Sigma, St Louis, MO; 200  $\mu\text{g}$  in 10% ethanol in 0.1 ml peanut oil) 48 h before they were killed by cervical dislocation.

Male mice were anaesthetized before surgical procedures with Avertin (1 mg  $\text{ml}^{-1}$  tribromoethyl alcohol in tertiary amyl alcohol (Sigma) diluted to 2.5% (v/v) in saline; 15  $\mu\text{l}$   $\text{g}^{-1}$  body mass injected i.p.). Mice were vasectomized by bilateral ligation of the vas deferens through a transverse incision in the abdominal wall (Hogan *et al.*, 1986), and seminal vesicles were removed through a transverse incision in the abdomen after ligation and severing of the proximal tubule at the base of the gland. The body wall and skin were sutured and the mice were allowed to recover for at least 2 weeks before mating.

#### Reagents and media

Media used for cell line and primary cell culture were RPMI-1640 or DMEM (Gibco, Grand Island, NY) supplemented with 20 mmol Hepes  $\text{l}^{-1}$  (Sigma), 10% (v/v) fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne),  $5 \times 10^{-5}$  mol  $\beta$ -mercaptoethanol  $\text{l}^{-1}$  and antibiotics (RPMI-FCS and DMEM-FCS, respectively). Recombinant *E. coli*-derived mouse GM-CSF was a gift from N. Nicola (Walter and Eliza Hall Institute of Medical Research, Melbourne).

#### Collection of uterine luminal fluid

Uterine intraluminal fluid was collected essentially as described by Robertson and Seamark (1990). Mice were killed by cervical dislocation between 10:00 h and 12:00 h on day 1 of pregnancy or pseudopregnancy and individual uterine horns isolated by cutting near the oviduct and the cervix. A blunt 26-gauge needle attached to a 1 ml syringe containing RPMI-FCS was then inserted into the oviductal end of a uterine horn and uterine washings (25–50  $\mu\text{l}$  in 500  $\mu\text{l}$  RPMI-FCS per horn) collected via the cervical end. For cytokine bioassay, flushings were centrifuged at 400 g for 10 min and the supernatant was stored at  $-80^\circ\text{C}$ . Total leucocyte counts in luminal flushings were made using a haemocytometer, and cell smears for haematoxylin and eosin staining of neutrophils were made from cell pellets after centrifugation of flushings at 400 g for 10 min.

#### Cytokine bioassays

GM-CSF in uterine fluids or cell culture supernatants was quantified by measuring proliferation of the specifically

GM-CSF dependent cell line FD 5/12 (Duhrsen, 1988) as described by Robertson and Seamark (1990). Standard curves were constructed using recombinant mouse GM-CSF. The SEM for dilutions used in data calculation in the cytokine bioassay were approximately 10%. Supernatants generated within the same experiment were assayed at the same time. Minimum detectable amounts were  $1 \text{ U ml}^{-1}$  for GM-CSF (50  $\text{U ml}^{-1}$  is defined as the concentration of GM-CSF stimulating half-maximal FD 5/12 cell proliferation, equivalent to 50 Colony Forming Units (CFU)  $\text{ml}^{-1}$  of recombinant GM-CSF, where 50 CFU  $\text{ml}^{-1}$  stimulates half-maximal colony development in a bone marrow assay).

#### Immunohistochemistry

Monoclonal antibodies (mAb) used were F4/80 (anti-macrophage; American Type Culture Collection (ATCC), Rockville, MD) (Austyn and Gordon, 1981) and RB6-8C5 (anti-neutrophil; provided by S. McColl, Department of Microbiology and Immunology, University of Adelaide) (Conlan and North, 1994). Eosinophils were detected by staining for endogenous peroxidase activity, which co-localizes with the eosinophil-specific stain Congo red and is absent from neutrophils in sections of uterus (S. Robertson, unpublished). Uterine tissue was embedded in OCT Tissue-Tek (Miles Scientific, Elkhart, IN) and frozen in isopropanol cooled by liquid  $\text{N}_2$ , then stored at  $-80^\circ\text{C}$  until used. Semi-serial sections (6  $\mu\text{m}$ ) taken from uteri of mice at oestrus or at day 1 of pregnancy were fixed in acetone (for RB6-8C5) or 96% ethanol (for F4/80 and endogenous peroxidase staining). For mAb staining, sections were incubated in humidified chambers in mAbs (neat hybridoma supernatant containing 10% normal mouse serum (NMS)) for 3 h at  $4^\circ\text{C}$  washed and incubated in goat  $\alpha$  rat-horseradish peroxidase (HRP; Dakopatts, Copenhagen; 1:20 in PBS containing 10% NMS for 2 h at  $4^\circ\text{C}$ ). Slides were incubated in diaminobenzidine (Sigma) (5 mg  $\text{ml}^{-1}$  in 0.05 mol Tris-HCl  $\text{l}^{-1}$ , pH 7.2) plus 0.02% (w/v) hydrogen peroxide for 10 min at room temperature to visualize HRP or endogenous peroxidase. Tissue was counterstained in haematoxylin. The numbers of neutrophils  $\text{mm}^{-2}$  luminal epithelium, or the area of positive staining in the endometrial stroma (expressed as a function of the area of total staining, after subtraction of the corresponding endogenous peroxidase value for sections stained with mAbs) were determined by Video Image Analysis using VideoPro software (Faulding Imaging, Adelaide).

#### Collection of male accessory gland fluid

In initial experiments, the secretions of the seminal vesicles, prostate and coagulating glands of adult male mice were collected immediately after death by excising the appropriate gland and dispersing the contents and tissues in 1.0 ml RPMI per gland or pair of glands with the aid of fine needles. Epididymal secretions were collected by flushing the ampullary region of the epididymis with 1.0 ml RPMI using a blunt 26-gauge needle. All preparations were pelleted at 5000 g for 15 min to remove cell debris and spermatozoa; supernatants were then collected and stored at  $-80^\circ\text{C}$  until cytokine assay.

For chromatography and other experiments, as indicated in the text, approximately 100  $\mu$ l seminal vesicle fluid was expressed from the intact, excised gland directly into 6 mol guanidine HCl  $l^{-1}$  (at a 1:4 v/v ratio).

### Chromatography

Fluid from the seminal vesicles of four 4–6-month-old stud male mice was pooled in 6 mol guanidine HCl  $l^{-1}$  (1:4 v/v). This pooled material was used for molecular sizing by chromatography or desalted by passage of 500  $\mu$ l through a 5 ml HiTrap column (Sephacrose G-25; Pharmacia, Uppsala) equilibrated in DMEM. The first 15 ml of eluate was assayed for GM-CSF stimulating activity. For molecular sizing chromatography, approximately 1 ml seminal vesicle fluid in 6 mol guanidine HCl  $l^{-1}$  was applied to a Sephacryl S-400 column (100 cm  $\times$  16 mm; Pharmacia) equilibrated in 6 mol guanidine HCl  $l^{-1}$ . Fractions of 15 ml were collected, desalted into DMEM and assayed for GM-CSF-stimulating activity.

For experiments investigating the nature of GM-CSF-stimulating activity, fractions were treated with trypsin (Sigma; 5 mg  $ml^{-1}$ , 30 min at 37°C) and then centrifuged through 100 000  $M_r$  cut-off microconcentrators (Amicon, Danvers, MA) before assay. Other fractions were extracted with acetone (70% v/v, -20°C for 60 min), or heated to 50°C or 80°C for 10 min before GM-CSF bioassay.

### Endometrial cell culture and assay of GM-CSF

Endometrial cells were prepared from mice in oestrus under sterile conditions using a modification (Robertson and Seamark, 1990) of a procedure described originally by Sherman (1978). Monolayers resulting from these preparations are composed of approximately 70% epithelial cells, 25% stromal fibroblasts and 3–5% leucocytes (Robertson *et al.*, 1992). Briefly, uteri from individual mice were excised, trimmed and slit lengthwise before incubation in 0.5% (w/v) trypsin, 2.5% (w/v) pancreatin (bovine pancreatic, type III, Sigma) in PBS for 45 min at 4°C followed by 45 min at 37°C. DMEM-FCS was added to neutralize proteases and uteri were gently agitated by repeated pipetting to release the epithelium and some underlying stromal cells. The yield for endometrial preparations was 7–14  $\times 10^5$  cells per uterus. Cells obtained from pools of four to six uteri were plated at approximately  $2 \times 10^5$  cells  $ml^{-1}$  in 1 ml DMEM-20% (v/v) FCS in four-well multidishes (Nunc, Roskilde), and incubated at 37°C in 5% CO<sub>2</sub> in air. Crude preparations or fractions from chromatographed seminal vesicle fluid in DMEM were added 3 h later at a 1:2 dilution to duplicate wells and incubated for 16 h when the supernatants were removed and replaced with fresh media, and the morphology of the cultured cells was examined under a microscope. After an additional 24 h culture period, supernatants were collected, cleared by centrifugation at 2000  $g$  for 5 min, and stored at -80°C before cytokine bioassay. Viable, adherent cells were quantified by measuring absorbance at 540 nm after incubation with Rose Bengal dye (Faulding, Adelaide) (0.25% w/v in PBS, 5 min at 20°C) and lysis in 1% (w/v) SDS, to enable expression of cytokine production as U  $10^{-5}$  cells.

### Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from whole uterine tissue according to the method of Chomczynski and Sacchi (1987). First strand cDNA synthesis was achieved by reverse transcription (RT) of RNA primed with random hexamers (Bresatec, Adelaide) using a Superscript RNase H-reverse transcriptase kit (Gibco) essentially according to the manufacturer's instructions and as detailed by Robertson *et al.* (1996). Quantification of mRNA by RT-PCR was performed essentially as described by Chelly *et al.* (1988), using primer pairs specific for GM-CSF and actin cDNAs which generate 278 bp and 186 bp products from cDNA, respectively, and reagents supplied in a Taq DNA polymerase kit (Bresatec) in 96-well PCR trays (Hybaid, Teddington), as detailed by Robertson *et al.* (1996). Each reaction included 0.1  $\mu$ Ci (approximately 3000 Ci  $mmol^{-1}$ ) [<sup>32</sup>P]dCTP (Bresatec). For amplification of GM-CSF and actin cDNAs, PCR was for 28–42 cycles and 14–28 cycles, respectively, at two cycle intervals. After photography of ethidium bromide-stained reaction products, gels were transferred by rapid alkaline transfer to nylon membrane (Schleier and Scheull, Dassel, Germany) and quantified using a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). The PCR product yield was plotted against reaction cycle as a semi-logarithmic curve. For estimation of the initial amount of the template, regression equations of the form  $y = 1 \times E^n$ , where  $y$  is the yield and  $n$  is the number of cycles, were fitted to the data in the linear portion of the curves using Sigma Plot software (Jandel Scientific Software, CA) as described by Robertson *et al.* (1996).

### Statistical analyses

Non-parametric tests were used in data analysis since in many experiments the data covered a broad range of values and were not normally distributed. Data were subjected to Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed) to evaluate differences between treatment groups. Differences were considered significant when  $P < 0.05$ .

### Results

#### Effect of male accessory gland deficiency on the GM-CSF response after mating

Previous studies using intact and vasectomized males in syngeneic and allogeneic mating combinations have shown that the GM-CSF-stimulating activity in semen was not associated with spermatozoa or histocompatibility antigens, thus implicating factors originating in the accessory glands (Robertson and Seamark, 1990). The extent to which the seminal vesicle fluid contributed to this activity was established by determining the GM-CSF content of luminal fluid in females 12 h after mating with intact, vasectomized, or seminal vesicle-deficient males.

Luminal fluid from mice mated with the seminal vesicle-deficient males was found to contain significantly less GM-CSF than that obtained from mice mated with intact or vasectomized males (median (95% confidence range) =  $< 1$  ( $< 1$ –

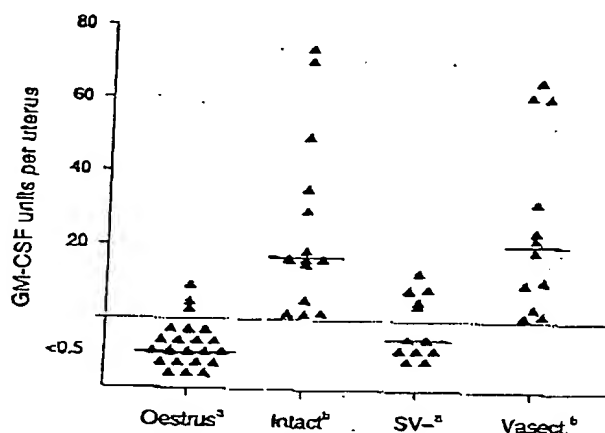


Fig. 1. The amount of granulocyte-macrophage colony stimulating factor (GM-CSF) in uterine luminal fluid of mice mated with intact, vasectomized or seminal vesicle-deficient males. The GM-CSF content of luminal fluids collected by lavage from mice at 10:00 h–12:00 h on the day of oestrus ( $n=22$ ), or on the day after mating with intact ( $n=17$ ) or vasectomized males (vasect;  $n=12$ ) or males from which the seminal vesicle glands had been surgically removed (SV-;  $n=12$ ) were assessed in the FD-5/12 bioassay. Symbols (▲) represent data from individual animals and median values are scored. Symbols below the dotted line have a cytokine content below the limit of detection for the bioassay. Data were compared by Kruskal-Wallis one-way ANOVA followed by Mann-Whitney Rank Sum (two-tailed) test. Different letters denote significant differences between treatments ( $P < 0.005$ ).

6.4) U per uterus for seminal vesicle-deficient males versus 8.1 ( $<1$ –36.8) U per uterus for intact males and 11.6 ( $<1$ –32.6) U per uterus for vasectomized males,  $P=0.001$ ; the amount being comparable with that recovered from uteri of unmated, oestrous females ( $<1$  ( $<1$ –4.7) U per uterus;  $P=0.123$ ) (Fig. 1). These data confirm previous experiments showing that GM-CSF-stimulating activity is not associated with spermatozoa or epididymal secretions (Robertson and Seamark, 1990), and suggest that this activity is due to a seminal vesicle-derived component of the ejaculate.

#### Effect of mating with intact and seminal vesicle deficient males on synthesis of mRNA encoding GM-CSF in vivo.

The amount of RNA encoding GM-CSF was assessed in the uteri of mice at oestrus and mice at days 1–4 of pregnancy to determine whether the seminal vesicle fluid-mediated increase in the luminal fluid content of GM-CSF bioactivity reflects the release of intracytoplasmic stores of protein or *de novo* synthesis by uterine tissues. A quantitative RT-PCR method was used since it was difficult to detect mRNA encoding GM-CSF in whole uteri from nonpregnant mice by northern blot analysis (data not shown). Total cellular RNA was extracted from whole uteri and analysed by quantitative RT-PCR for mRNA encoding GM-CSF and actin. mRNA encoding actin was found to be independent of treatment, with a coefficient of variation of less than 20% between each preparation (data not shown). In contrast, the uterine content of mRNA encoding GM-CSF of uteri varied with day of pregnancy (Fig. 2), with a

peak in expression on day 1 of pregnancy when values were 5.7 times greater than those found at oestrus. Expression remained high on day 2, but declined thereafter to a value on day 4 of 1.6 times that at oestrus.

Total cellular RNA was extracted from uteri of mice at oestrus and from mice mated with intact or seminal vesicle-deficient males ( $n=6$  per group), and the mRNA encoding GM-CSF and mRNA encoding actin content quantified to determine whether depletion of seminal vesicle fluid from the ejaculate was associated with diminished GM-CSF expression after mating. Expression in uteri of mRNA encoding GM-CSF in mice mated with intact males was significantly greater than that in uteri from mice at oestrus or from mice mated with seminal vesicle-deficient males (median (95% confidence range) = 77 (24–340)% median oestrous value for matings with seminal vesicle-deficient males, versus 920 (260–3400)% median oestrous value for intact males,  $P=0.002$ ) (Fig. 3). These data indicate that release of GM-CSF after mating is accompanied by an increase in the synthesis of mRNA encoding GM-CSF, and furthermore, provide evidence that this upregulation in synthesis is dependent on the activity of factors in the secretions from the seminal vesicles.

#### Effect of seminal vesicle deficiency on the inflammatory response after mating

Immunohistochemical analyses with mAbs specific for leucocyte common antigen (CD45) and myeloid lineage-specific antigens in this and other laboratories have shown that the leucocytes resident in the cyclic and early pregnant uterus are predominantly of macrophage, granulocyte and dendritic cell lineages (De *et al.*, 1991; Kachkache *et al.*, 1991; McMaster *et al.*, 1992; S. Robertson, unpublished). Uteri were collected from mice at oestrus and approximately 12 h after mating with intact, vasectomized or seminal vesicle-deficient males, and the composition and number of endometrial leucocytes were investigated immunohistochemically using F4/80 and RB6-8C5 mAbs specific for macrophages and neutrophils, respectively, and endogenous peroxidase activity to detect eosinophils to determine whether the infiltration of inflammatory leucocytes on day 1 of pregnancy is dependent on the seminal vesicle-derived components of the ejaculate. The number and distribution of macrophages and granulocytes in the endometrium were found to be dependent on exposure to semen, despite substantial variability in the densities of both leucocyte populations within groups of both oestrous and mated mice.

In the uterus of mice at oestrus, F4/80<sup>+</sup> macrophages were distributed throughout the endometrial stroma, with endogenous peroxidase-positive eosinophils accumulated predominantly at the endometrial-myometrial junction and also scattered throughout the endometrial stroma (Fig. 4). The dynamics of the inflammatory response after mating in mice mated with intact or vasectomized males were similar to those reported previously by others (De *et al.*, 1991; Kachkache *et al.*, 1991; McMaster *et al.*, 1992), with mating causing a significant increase in the density of macrophages within the endometrial stroma immediately subjacent to the luminal surface (Table 1). A shift in the position of eosinophils from the endometrial-myometrial junction to areas of the endometrium underlying

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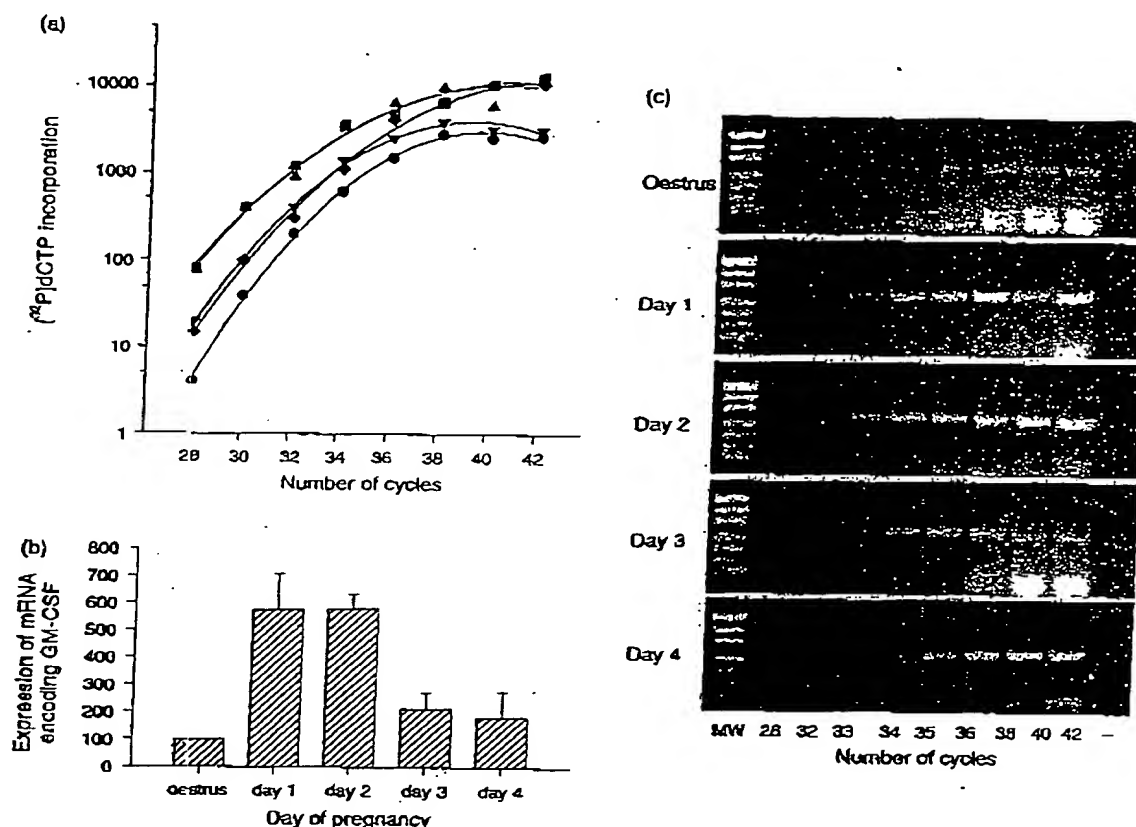


Fig. 2. Quantitative reverse transcriptase (RT)-PCR analysis of the expression of RNA encoding granulocyte-macrophage colony stimulating factor (GM-CSF) during early pregnancy. Total cellular RNA was extracted from intact uteri collected from mice at 10:00 h–12:00 h on the day of oestrus or on days 1, 2, 3 or 4 of pregnancy after mating with intact males ( $n = 2$  pooled per time point). RNA preparations were subjected to reverse transcription by random priming and amplified by PCR with GM-CSF or actin-specific primers for various numbers of cycles. PCR products were subjected to electrophoresis on agarose gels and photographed on a UV-transilluminator (c). The amount of product was quantified by phosphor-imaging after transfer of products to nylon membrane and plotted as a function of cycle number (a). Data were analysed by linear regression analysis to calculate the relative contents of mRNA encoding GM-CSF of each RNA preparation, normalized to actin content. The mean  $\pm$  SD contents of mRNA encoding GM-CSF is expressed as a percentage of values at oestrus ( $n =$  three experiments) and is shown in (b).

the epithelium was notable after mating with intact males, and to a lesser degree after mating with vasectomized males. Mating with either intact or vasectomized males was also associated with a striking infiltration of RB6-8C5<sup>+</sup> neutrophils which were sparsely distributed at oestrus but comparable with eosinophils in number on day 1 of pregnancy (Fig. 5). After mating, many neutrophils were also interspersed between epithelial cells at the luminal surface, where they appeared to be in the process of moving into the luminal cavity.

Differences were evident in the composition of the leucocyte population infiltrating the endometrium after matings with seminal vesicle-deficient males compared with those found after mating with intact or vasectomized males. Most notable was a marked reduction in the number of neutrophils. The pattern of RB6-8C5 staining in the endometrium of mice mated with seminal vesicle-deficient males was indistinguishable from that of mice at oestrus, with neutrophils sparsely distributed in the endometrium and none detectable in the epithelial layer (Table

1, Fig 5). There was also a trend towards fewer macrophages in the subepithelial endometrial stroma of mice mated to seminal vesicle-deficient males, but this difference was not significant.

The number of neutrophils in the luminal fluid of mice collected by lavage approximately 12 h after mating with intact and seminal vesicle-deficient males was determined to investigate further the effect of seminal vesicle deficiency on the efflux of neutrophils into the uterine lumen. Neutrophils were abundant in the luminal fluid of mice mated with intact males (Table 1), constituting > 95% of the leucocytes present and occurring primarily in large aggregates with spermatozoa. In contrast, the luminal fluid of mice mated with seminal vesicle-deficient males contained fewer leucocytes and proportionately fewer neutrophils, with individual spermatozoa remaining mono-dispersed (Fig. 5). These data confirm that the seminal vesicle-derived component of the ejaculate has an important role in provoking the cellular changes that characterize the inflammatory response after mating, and are



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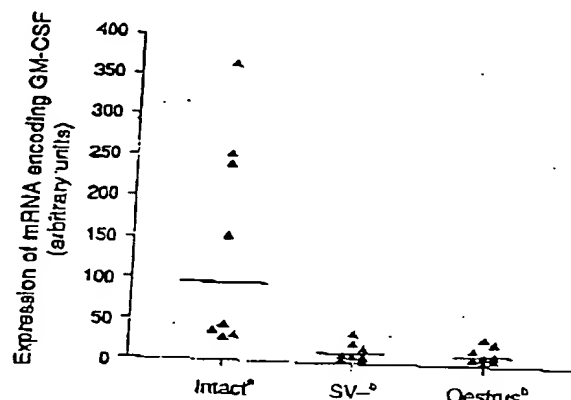


Fig. 3. The effect of seminal vesicle deficiency on uterine expression of granulocyte-macrophage colony stimulating factor (GM-CSF). The relative content of mRNA encoding GM-CSF of individual uteri collected from mice at 10:00 h–12:00 h on the day of oestrus ( $n=8$ ), or on the day after mating with intact males ( $n=8$ ) or males from which the seminal vesicle glands had been surgically removed (SV-;  $n=8$ ) was determined as described in Fig. 2. Each symbol ( $\Delta$ ) represents the amount of mRNA encoding GM-CSF in arbitrary units (normalized to the content of mRNA encoding actin) per uterus, and median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. Different letters denote significant differences between treatments ( $P<0.002$ ).

consistent with a key role for upregulated GM-CSF expression induced by seminal factors in initiating these events.

#### Effect of male accessory gland fluids on GM-CSF synthesis *in vitro*

A series of *in vitro* studies was carried out to determine the extent to which secretions from the other accessory glands and organs contribute to the flux in uterine GM-CSF. The capacity of fluids from the seminal vesicles, prostate, coagulating gland and epididymis of 4–6-month-old stud males to stimulate GM-CSF release from endometrial cells collected from the pooled uteri of mice at oestrus was examined. Epithelial cells comprise >70% of the endometrial cell preparations used in these experiments and have been identified as the exclusive source of GM-CSF synthesized in these cultures (Robertson *et al.* 1992). GM-CSF output from endometrial cells was significantly enhanced ( $7.6 \pm 0.2$  fold increase) in cultures exposed to seminal vesicle fluid compared with control cultures, whereas inclusion of fluids from the coagulating or prostate glands or epididymis had no significant effect (Fig. 6), confirming the finding *in vivo* that the seminal vesicle is the predominant source of GM-CSF stimulating activity in the ejaculate.

A series of additional experiments was carried out *in vitro* to optimize methods for maximum recovery of the GM-CSF stimulating activity from seminal vesicle glands. Cytotoxic effects accompanied by loss of GM-CSF-stimulating activity were noted following a delay of more than one minute in excision of the seminal vesicles after exsanguination, contamination with coagulating gland fluid during excision, or inad-

vertent aeration of vesicle fluid during maceration which caused seminal vesicle fluids to precipitate. Maximal stimulation of GM-CSF secretion was achieved after 16 h (compared with 4 h or 8 h) co-incubation of seminal vesicle extract with endometrial cells (data not shown). In six experiments, the median (95% confidence range) increase in endometrial cell GM-CSF output after incubation with precipitate-free seminal vesicle extracts was 320 (150–760)% ( $P<0.05$ ).

#### Effect of age, sexual experience and testosterone status on GM-CSF stimulating activity

To determine whether age, sexual experience and testosterone status influence the GM-CSF stimulating capacity of male mice (and to identify an appropriate population of animals to use as the source of material for further characterization of the nature of the stimulating factor(s)), seminal vesicle fluid was collected from 3-month-old male mice that had been caged either in groups ('unseparated') or individually for 3 weeks, and from 4–6-month-old males that had been caged individually and had been used as stud males on a regular basis for at least 2 months. In addition, seminal vesicle fluid was collected from unseparated 3-month-old male mice that had been given injections of 200  $\mu$ g testosterone 48 h before being killed in an effort to increase their yield of GM-CSF stimulating factor. Excised glands were weighed immediately before maceration. Fluids were incubated with endometrial cells as described above, and measurements were made of the amount of GM-CSF released per  $10^5$  adherent endometrial cells.

The GM-CSF-stimulating activity of seminal vesicle fluids was highly variable and differed between treatment groups. Stud males had significantly larger seminal vesicles (mean  $\pm$  SD =  $254 \pm 35$  mg;  $P=0.001$ ) than unseparated or inexperienced males, and their seminal vesicle fluid contained significantly more GM-CSF stimulating activity than all other groups (median ( $\pm$  95% confidence range) GM-CSF output = 233 (119–439)% control;  $P=0.001$ ) (Fig. 7). Caging inexperienced male mice individually significantly increased both the mass of seminal vesicles (unseparated:  $127 \pm 31$  mg versus separated:  $183 \pm 18$  mg;  $P=0.001$ ), and the content of GM-CSF stimulating activity (unseparated: 77 (27–156)% versus separated: 127 (86–318)%;  $P=0.021$ ). Treatment of mice with testosterone increased the mean ( $\pm$  SD) mass of seminal vesicles in unseparated males ( $236 \pm 16$  mg;  $P=0.001$ ), but inhibited GM-CSF stimulating activity to values significantly below that of all other groups (33 (13–176)%;  $P=0.010$ ). The increase in the mass of seminal vesicles would have contributed to, but does not fully account for the increased GM-CSF stimulating activity found in experienced and separated males, suggesting that sexual status influences both the total content and the content relative to gland mass of seminal vesicle GM-CSF stimulating activity.

#### Molecular mass and proteinaceous nature of GM-CSF stimulating activity

Preliminary experiments established that GM-CSF stimulating activity could be recovered after dissolution of seminal vesicle fluid in 6 mol guanidine HCl  $l^{-1}$  and desalting using

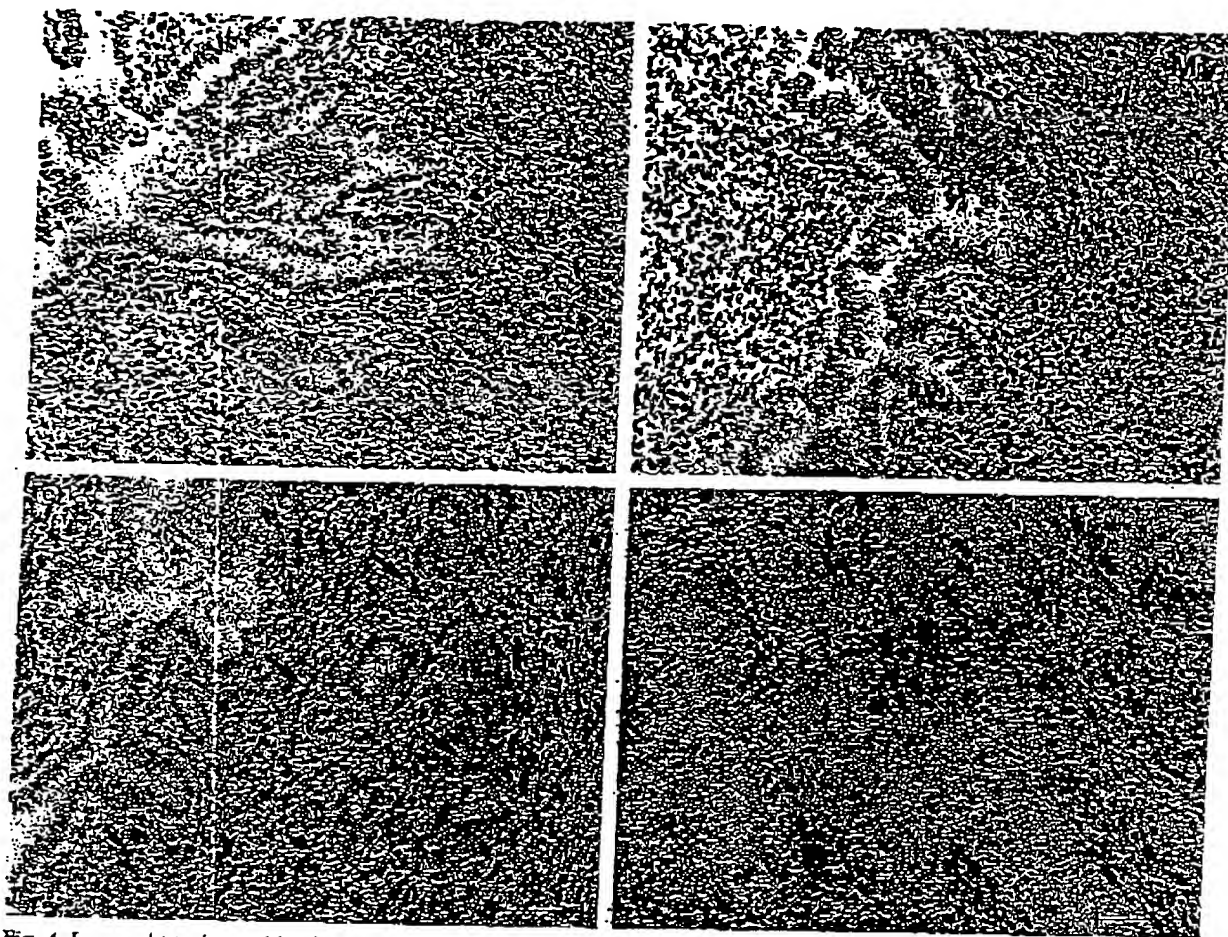


Fig. 4. Immunohistochemical localization of F4/80 + macrophages and endogenous peroxidase plus eosinophils in the uteri of mice killed at 10:00 h–12:00 h on the day of oestrus, or on the day after mating with intact males. Endogenous peroxidase plus eosinophils (small intensely stained cells) predominate at the endometrial-myometrial junction at oestrus (small arrows) (a) and are more plentiful in the endometrium after mating (b). Macrophages (large stained cells) are more abundant in the subepithelial endometrial stroma after mating (d) than at oestrus (large arrows) (c). Scale bars represent 100 µm. Ep: epithelium; En: endometrium; My: myometrium.

Sephacryl S-400 column. On each of five occasions, the major proportion of GM-CSF-stimulating activity was found to elute in a peak corresponding to approximately 650 000 M<sub>r</sub>, with a lesser and more heterogeneous peak of activity eluting in the 100 000–400 000 M<sub>r</sub> range (Fig. 8). The bioactive fractions were desalted onto Sepharose G-25 and subjected to various protein-denaturing

treatments. The activity was found to be heat sensitive, with no activity and < 50% activity remaining after 10 min incubation at 80°C and 60°C, respectively. Incubation with trypsin or extraction with acetone, but not ether, also completely abolished the GM-CSF-stimulating activity, suggesting that the active factor was wholly, or partially, composed of protein.

### Discussion

During the oestrous cycle in mice, ovarian steroid hormones regulate the secretion from uterine epithelial cells of a family of lymphohaemopoietic cytokines including GM-CSF, CSF-1, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and an eosinophil chemotactic factor (ECF-U), which together orchestrate the recruitment and regulate the function of macrophages and eosinophils in the mouse endometrial stroma (Robertson and Seamark 1992; Robertson *et al.* 1995; Lee *et al.* 1989). The present study identifies a high molecular mass, proteinaceous component of

Table 1. The effect of mating with intact and seminal vesicle-deficient males on the numbers of neutrophils, eosinophils and macrophages in the endometrial stroma, luminal epithelium and luminal cavity of uteri of mice

Parameter	Units	Values at oestrus	Males		
			Intact	Vasectomized	Seminal vesicle-deficient
Neutrophils:					
Endometrial stroma <sup>a</sup>	Percentage positivity	9.5 (2.7–14) <sup>d</sup>	23 (7.5–41) <sup>c</sup>	50 (20–65) <sup>c</sup>	4.7 (2.6–13) <sup>de</sup>
Epithelium <sup>b</sup>	Cells mm <sup>-2</sup> epithelium	<0.1 (<0.1) <sup>d</sup>	4.9 (0.3–12) <sup>c</sup>	3.5 (<0.1–16) <sup>d</sup>	0.1 (<0.1–1.2) <sup>de</sup>
Luminal cavity <sup>c</sup>	Cells ( $\times 10^{-4}$ ) per uterus	0.3 (<0.1–5.0) <sup>d</sup>	22 (2.0–250) <sup>c</sup>	—	0.5 (<0.1–1.2) <sup>d</sup>
Macrophages <sup>e</sup>	Percentage positivity	15 (12–25) <sup>d</sup>	26 (15–49) <sup>d</sup>	42 (18–49) <sup>c</sup>	20 (9–32) <sup>de</sup>
Eosinophils <sup>f</sup>	Percentage positivity	4.2 (1.6–8.1) <sup>d</sup>	9.7 (5.1–19) <sup>c</sup>	3.5 (2.9–6.8) <sup>de</sup>	10 (2.7–14) <sup>de</sup>

Data are from at least six mice per group.

<sup>a</sup>Leucocytes in the endometrial stroma were detected by immunohistochemical analysis using RB6-8C5 and F4/80 mAbs or no mAb (to detect neutrophils, macrophages and endogenous peroxidase plus eosinophils, respectively) on sections of uteri from mice killed at 10:00 h–12:00 h on the day of oestrus or on the day after mating with intact, vasectomized or seminal vesicle-deficient males. The area of mAb reactivity in sections was determined using Vidco Pro Image Analysis Software as described in the Materials and Methods, and data are given as median (95% confidence range) percentage positivity.

<sup>b</sup>Neutrophils in the epithelium were detected by immunohistochemical analysis using RB6-8C5 and values are the median (95% confidence range) number of neutrophils mm<sup>-2</sup> epithelium.

<sup>c</sup>Neutrophils in the luminal cavity were detected by haematoxylin and eosin staining of smears of cells recovered by lavage and values are the median (95% confidence range) number ( $\times 10^{-4}$ ) of neutrophils per uterus.

<sup>d</sup>Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum (two-tailed) test. Different letters denote significant differences between treatments ( $P < 0.05$ ).

seminal plasma which acts, at mating, to initiate the post-mating inflammatory cascade by upregulating the synthesis and release of GM-CSF from oestrogen-primed epithelial cells lining the uterine lumen. The resultant accumulation of GM-CSF in uterine fluids is accompanied by a marked increase in the recruitment and activation of macrophages, neutrophils and eosinophils within the endometrial stroma subjacent to the luminal surface. Large numbers of neutrophilic granulocytes migrate through the basement membrane, between epithelial cells and into the luminal cavity where they form complex aggregates with spermatozoa.

An efflux of endometrial leucocytes into the uterine luminal cavity after mating termed the 'leucocytic cell reaction' (reviewed by Barratt *et al.*, 1990) has been described in rats and mice (Austin, 1957, 1975), and a comparable leucocytic response occurs in the cervix in other species including humans (Moyer *et al.*, 1970; Pandya and Cohen, 1985) and rabbits (Phillips and Mahler, 1975; Tyler, 1977) where semen is deposited in the vagina. These leucocytes have been judged, on the basis of their morphology, to be mainly neutrophils with small numbers of macrophages and lymphocytes, and this has been confirmed with specific mAbs in women (Thompson *et al.*, 1992). Copulation is also associated with an effusion of immunoglobulin into the cervix or uterine luminal cavity which mediates the opsonization and subsequent phagocytosis of the vast majority of spermatozoa (reviewed by Cohen, 1984). In rabbits and humans, studies involving mating with vasectomized males or application of seminal plasma to the cervix suggest that spermatozoa are necessary for eliciting these responses (Tyler, 1977; Pandya and Cohen, 1985; Thompson *et al.*, 1992).

In the present study, it was found that the plasma rather than the sperm component of semen evoked the inflammatory response after mating and 'leucocytic cell reaction' in mice. In females mated with vasectomized males, the release of

GM-CSF and ensuing leucocyte infiltrate were almost indistinguishable from that occurring after mating with intact males. In both cases, macrophages and neutrophils were recruited into the subepithelial endometrial stroma but only neutrophils were observed to migrate between epithelial cells and into the luminal space. The only apparent difference between the uterine response to mating with intact and vasectomized males was in the recruitment of eosinophils, suggesting that this component of the inflammatory cascade after mating may be influenced by sperm-associated factors.

In contrast, no increase in GM-CSF output from the epithelium occurred when mice were mated with seminal vesicle-deficient males. Furthermore, in the absence of seminal vesicle fluid the recruitment of granulocytes into the endometrium was diminished and there was a complete lack of neutrophil infiltration into the luminal cavity. These data provide compelling support for a causal relationship between GM-CSF release and leucocyte infiltration as previously suggested by experiments instilling exogenous GM-CSF into the reproductive tract (Robertson *et al.*, 1994) and studies in GM-CSF deficient transgenic mice (S. Robertson, unpublished). The reduction in numbers of neutrophils in the luminal fluid of mice mated to seminal vesicle-deficient males could not be accounted for by a reduced contribution from male-derived leucocytes present in the semen (Barratt *et al.*, 1990), since immunohistochemical staining of uterine sections at day 1 of pregnancy with mAb specific for paternal MHC class I showed that more than 99% of the leucocytes present in the luminal cavity after natural mating were of maternal origin (S. Robertson and K. Farr, unpublished). Seminal vesicle deficiency did not completely inhibit the recruitment of eosinophils into the endometrial stroma after mating, a finding which is consistent with a role for spermatozoa in regulating the movements of these cells. Further investigation is required to evaluate the effect of seminal vesicle deficiency on other potentially GM-CSF depen-

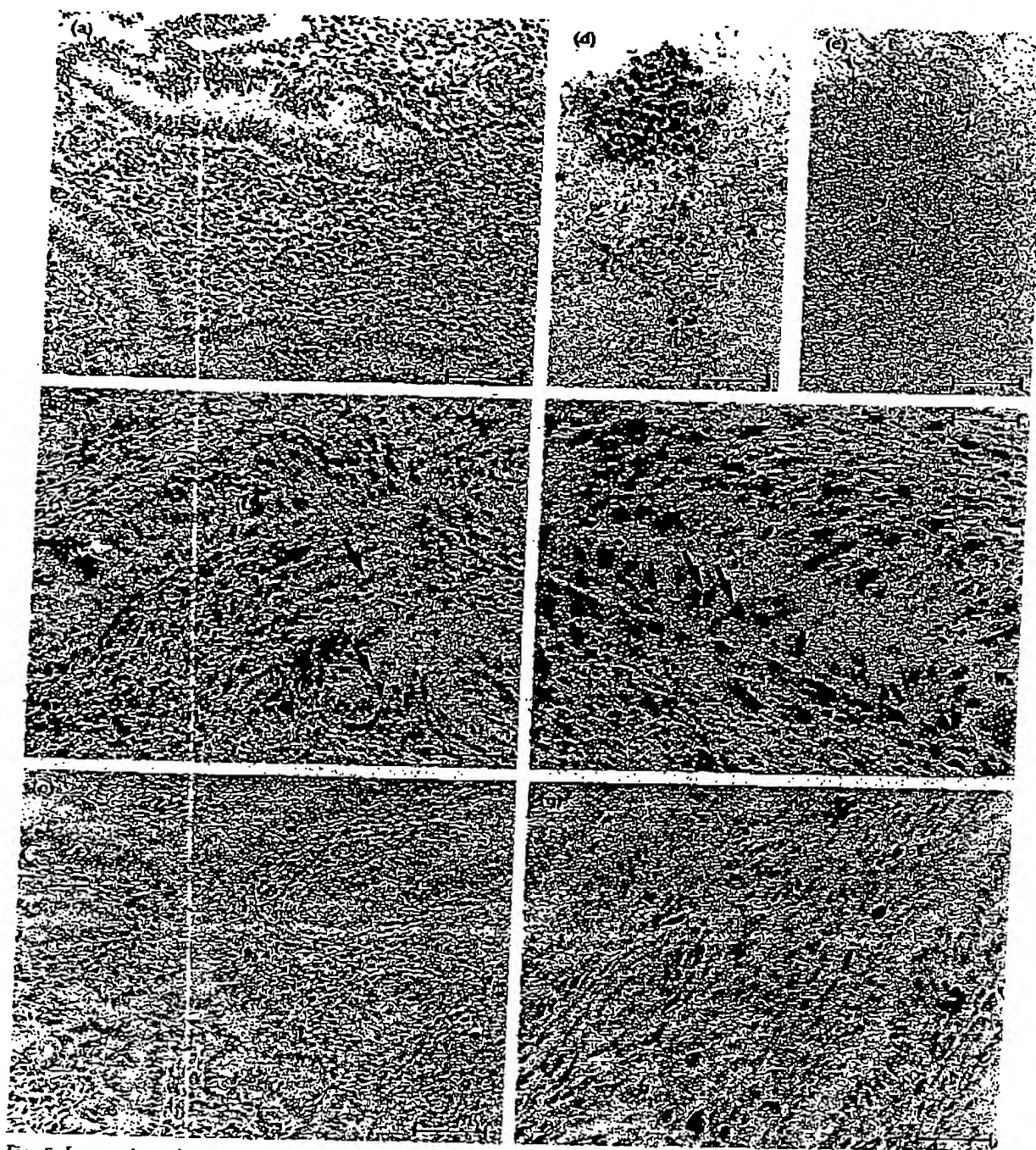


Fig. 5. Immunohistochemical localization of RB6-8C5 + neutrophils in the uteri of mice killed at 10:00 h–12:00 h on the day of oestrus (a) or on the day after mating with intact or seminal vesicle-deficient males. Numerous neutrophils (intensely stained cells; large arrows) are evident in the subepithelial endometrial stroma and between luminal epithelial cells after mating with intact males (b,f) but are scarce in the endometrium and absent from the epithelium of seminal vesicle-deficient males (c,g). Endogenous peroxidase plus eosinophil (small intensely stained cells; small arrows in (f)) are also evident in all sections. Smears of cells from uterine luminal fluid stained with haematoxylin and eosin show neutrophils in large aggregates with spermatozoa after mating with intact males (d) but not with seminal vesicle-deficient males (e). Scale bars (a)–(c): 100 µm; (d) and (e): 25 µm; (f) and (g): 50 µm.



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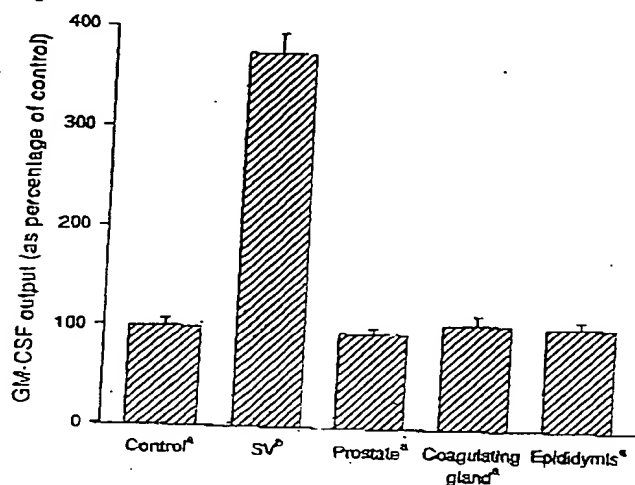


Fig. 6. Granulocyte-macrophage colony stimulating activity (GM-CSF) in seminal vesicle fluid *in vitro*. Accessory gland fluids were prepared from a 6-month-old stud mouse and cultured with oestrous endometrial cells for 16 h when the supernatants were replaced with fresh medium. The GM-CSF content of supernatants collected 24 h later is given as a percentage of the content of supernatants collected from endometrial cells cultured in medium alone. Different letters denote significant differences between treatments ( $P < 0.005$ ).

dent parameters including the activation state, secretory profile and efflux of macrophages and dendritic cells into the afferent lymphatics.

No evidence was obtained to implicate accessory glands other than the seminal vesicle in GM-CSF release. The potency of seminal vesicle fluid activity was shown to vary depending on the sexual status of the male, with active stud males having a superior capacity to enhance GM-CSF release from endometrial cells compared with young, inexperienced males. It is known that testosterone has a critically important role in regulating the size and function of the seminal vesicles, but the potency of activity from males caged in groups declined rather than improved after testosterone administration, suggesting that either the dose of testosterone chosen may have been inappropriate or that other regulating determinants may also be important. Individual differences in the seminal fluid content of GM-CSF stimulating activity may contribute to the considerable variability in GM-CSF synthesis and the extent of the inflammatory infiltrate that was evident in uteri from mice mated with both intact and vasectomized males. The precise stage of the oestrous cycle and systemic immune parameters in the female may be additional determinants of the intensity of the inflammatory response after mating.

A preliminary characterization of the nature of seminal GM-CSF stimulating activity indicates that it is primarily associated with a proteinaceous moiety of approximately 650 000  $M_r$ . In most experiments bioactivity was also associated with smaller molecules, which may or may not be related to the 650 000  $M_r$  moiety. Chromatographic purification of these proteins to homogeneity is currently underway, and future studies will investigate the interrelationship between the various peaks of bioactivity.

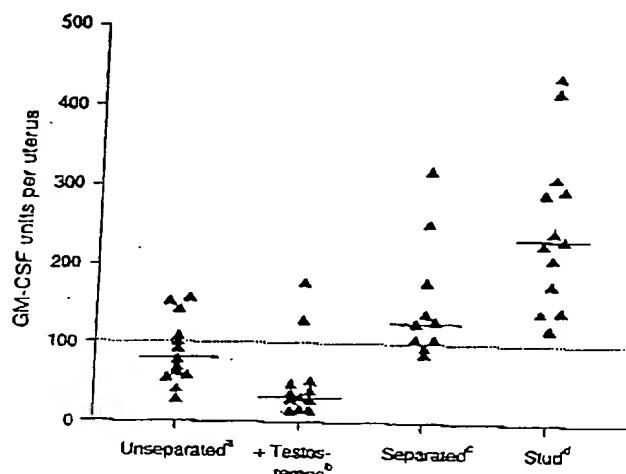


Fig. 7. Effect of testosterone status on the granulocyte-macrophage colony stimulating activity (GM-CSF) in seminal vesicle fluid. The GM-CSF output of endometrial cells from mice at oestrus after incubation with seminal vesicle fluid from testosterone treated (+ testosterone;  $n = 12$ ) untreated 2–3-month-old males kept in groups (unseparated;  $n = 13$ ), 2–3-month-old male mice separated for 3 weeks (separated;  $n = 10$ ) and 4–6-month-old male stud (stud;  $n = 13$ ) mice are plotted as a percentage of the output from endometrial cells cultured in medium alone. Symbols (A) represent data from individual animals and median values are scored. Symbols below the dotted line have a cytokine content below the limit of detection for the bioassay. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum (two-tailed) test. Different letters denote significant differences between treatments ( $P < 0.02$ ).

The sequence of events initiated by the action of semen on the uterine epithelium is analogous to the cytokine-mediated responses to inflammatory stimuli of other epithelia, including the skin and airways. In these tissues, an inflammatory response precedes and facilitates the generation of an effective immune response to potentially harmful initiating agents (Matzinger 1994). However, the physiological significance of the uterine response to semen, and its impact on the success of implantation and placental formation, remains a topic of speculation. In our preliminary experiments, pregnancies sired by seminal vesicle deficient males were less likely to result in viable implantations (four of ten mated mice had viable implantation sites at day 17 of pregnancy versus eight of eight for intact studs), with comparable litter sizes in pregnant females (S. Robertson, unpublished). Other studies in rodents show that although pregnancy can be initiated and may proceed in the absence of seminal plasma (Blandau, 1945), seminal vesicle deficiency in males severely restricts their fertility (Pang *et al.*, 1979; Queen *et al.*, 1981). The extent to which a dysregulated endometrial response to mating may be the cause of this reduction in fertility remains to be determined, since vaginal plug formation and hence retention of spermatozoa within the uterine cavity is diminished in the absence of seminal vesicle fluid (Carballada and Esponda, 1992). However, an additional role for seminal vesicle fluid was suggested by the finding that fertility was less severely affected when the coagulating glands

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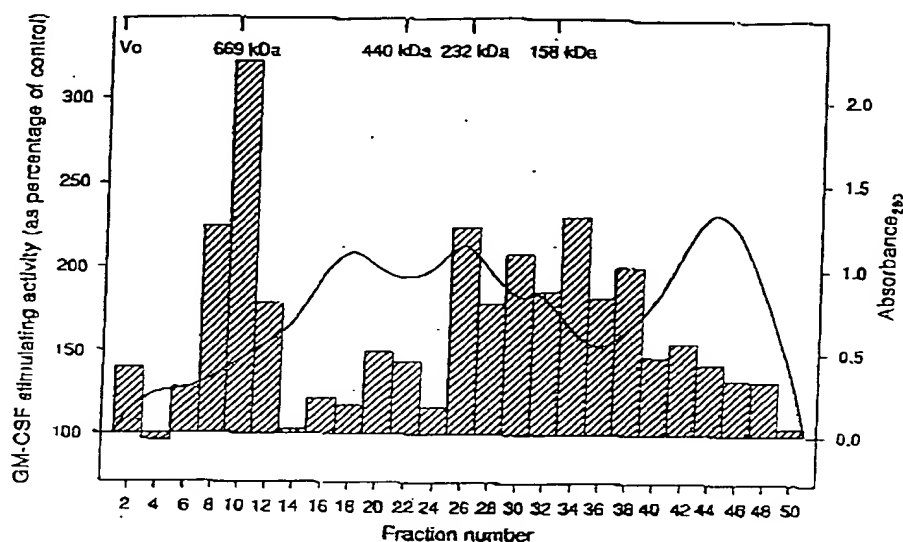


Fig. 8. Sephacryl S-400 size exclusion chromatography of granulocyte-macrophage colony stimulating activity (GM-CSF) from the seminal vesicles of mice. Fluid was collected from the seminal vesicles of 4–6-month-old stud mice and solubilized in 6 mol guanidine HCl l<sup>-1</sup>, and then subjected to chromatography through Sephacryl S-400. Eluted fractions were desalted over Sepharose G-25 into DMEM, cultured with oestrous endometrial cells for 16 h and then replaced with fresh medium. The GM-CSF content of supernatants collected 24 h later are given as a percentage of the content of supernatants collected from endometrial cells cultured in DMEM-FCS alone. The position of elution of molecular mass standards is shown at the top of the figure.

were removed (Pang *et al.*, 1979), despite a similar necessity for coagulating glands and seminal vesicles in vaginal plug formation.

The pre- and peri-implantation period of development is clearly of major importance in determining the outcome of pregnancy, but the roles of the GM-CSF-responsive, endometrial leucocyte populations in the events of early pregnancy remain to be fully elucidated. Neutrophils which infiltrate the luminal cavity would certainly have a 'housekeeping' role in clearing the uterus of redundant spermatozoa and micro-organisms introduced at mating, and the possibility that they are involved in sperm selection has also been canvassed (Cohen, 1984). However, we argue that the complexity of the inflammatory response after mating would afford the participating leucocytes a more pro-active role in the events leading to successful implantation and placentation. Macrophages and granulocytes recruited into the endometrial stroma are certainly well equipped to mediate in tissue remodelling events through the release of proteases and other enzymes, and are a potent source of a plethora of cytokines and immunoregulatory molecules that regulate the differentiation and function of leucocytes and cells of other lineages. Local macrophages and dendritic cells are clearly capable of sampling and processing seminal antigens, some of which are expressed by the placenta after implantation (Thaler, 1989), since significant changes in the cellularity of lymph nodes draining the uterus occur during the pre-implantation period of pregnancy (Beer and Billingham, 1974; Clarke, 1984). The nature and extent of immune responses in mucosal sites is highly responsive to the cytokine microenvironment (Husband *et al.*, 1994), presumably through influences on the phenotype of local antigen-presenting cells.

The effects of seminal plasma on local cytokine expression would thus be expected to be pivotal in eliciting the appropriate type of immune response to seminal antigens and in setting the stage for the immunobiology of pregnancy, perhaps through initiating T helper cell type 2 responses (Wegmann *et al.*, 1993) or T-cell tolerance to paternal major histocompatibility antigens (Tafari *et al.*, 1995). Indeed, support for the postulate that seminal plasma has a role in attenuating the immune response to spermatozoa in mice was provided by Beer and Billingham (1974), with an intact ejaculate eliciting hypertrophy of draining lymph nodes but not the transplantation immunity seen after sensitization by intrauterine inoculation of washed spermatozoa.

The extent to which these findings may be related to reproductive success in humans is unknown. Human uterine epithelial cells synthesize many of the same cytokines made in rodent uteri, including GM-CSF (Giacomini *et al.*, 1995; Robertson *et al.*, 1995). However, in humans, unlike rodents, the seminal material is deposited in the vagina and is largely excluded from the uterus by the cervix. Components of seminal plasma are known to infiltrate the uterine cavity in association with spermatozoa, but if immunological priming occurs in draining lymph nodes, then antigen sampling may occur in association with leucocytosis at the cervix. There is a body of evidence suggesting that cumulative exposure to seminal factors may specifically 'condition' the human reproductive tract for pregnancy (Marti and Herrmann, 1977; Need *et al.*, 1983; Serhal and Craft, 1987), which is now being re-examined following recent claims that the incidence of both pregnancy-induced hypertension and pre-eclampsia are inversely correlated with the length of sexual cohabitation before conception.

(Robillard *et al.*, 1995), except when barrier forms of contraception are used (Klonoff-Cohen *et al.*, 1989). Instillation of semen or sexual intercourse at the time of ovulation is also claimed to enhance the implantation rate of embryos developed by in gamete intrafallopian tube transfer (Marconi *et al.*, 1989) and by *in vitro* fertilization (IVF), possibly through an effect on the endometrium (Bellings *et al.*, 1986). The relative importance of spermatozoa and seminal plasma in achieving these effects has not been investigated, but the finding that insemination with 'split' ejaculates, which are deficient in vesicular secretions, does not benefit patients undergoing IVF (Fischel *et al.*, 1989) supports the postulate that seminal vesicle-derived proteins are essential for evoking an appropriate maternal response. Further studies aimed at more clearly defining the immunological consequences of the mating event and their potential contribution to pregnancy success are thus clearly warranted.

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## Medline Abstract



SA Robertson and RF Seamark

**Granulocyte macrophage colony stimulating factor (GM-CSF) in the murine reproductive tract: stimulation by seminal factors.**

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The activity of GM-CSF during early pregnancy in the murine uterine lumen in vivo and in media conditioned by uterine cells in vitro has been assessed. GM-CSF was detected in uterine luminal fluid recovered by lavage on the morning after syngeneic mating (median level 5.7 CFUc U/uterus) and following mating with vasectomized (5.1 U/uterus) or allogeneic males (4.4 U/uterus), with significantly lesser ( $P$  less than 0.05) amounts recovered from the uteri of superovulated, mated mice. By contrast, GM-CSF was only detectable (greater than 0.5 U/uterus) in the luminal fluid of three of 22 unmated oestrous mice examined. No activity was detected in secretions from male accessory glands including seminal vesicle, epididymis, prostate and coagulating gland (less than 0.5 U/gland). GM-CSF was found at higher levels in supernatants from cell monolayers prepared by tryptic digest of the uteri of Day 1 mated mice than those from unmated oestrous mice ( $P$  less than 0.05). Little GM-CSF was detected in supernatants from ovariectomized mice. An alpha-GM-CSF polyvalent antibody neutralized the FD5/12 bioassay response confirming the identity of the lymphokine. The interleukins IL-2 and IL-3 were not detected in uterine luminal fluid nor in media conditioned by cell monolayers. We postulate that elevated uterine GM-CSF activity after mating is elicited by a non-sperm associated, non-MHC component of the ejaculate and synthesized by a hormone-responsive endometrial cell population. This cytokine may have an embryotrophic role or contribute to priming of the uterus for implantation.

### Publication Type:

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### MeSH Terms:

- Animal
- Cell Line
- Cells, Cultured
- Culture Media
- Epididymis
- Female
- Granulocyte-Macrophage Colony-Stimulating Factor
- Male
- Mice
- Pregnancy
- Prostate
- Seminal Vesicles
- Support, Non-U.S. Gov't